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Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides

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The formation of inositol phosphates in response to agonists was studied in brain slices, parotid gland fragments and in the insect salivary gland. The tissues were first incubated with [^{3}H]inositol, which was incorporated into the phosphoinositides. All the tissues were found to contain glycerophosphoinositol, inositol 1-phosphate, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate, which were identified by using anion-exchange and high-resolution anion-exchange chromatography, high-voltage paper ionophoresis and paper chromatography. There was no evidence for the existence of inositol 1:2-cyclic phosphate. A simple anion-exchange chromatographic method was developed for separating these inositol phosphates for quantitative analysis. Stimulation caused no change in the levels of glycerophosphoinositol in any of the tissues. The most prominent change concerned inositol 1,4-bisphosphate, which increased enormously in the insect salivary gland and parotid gland after stimulation with 5-hydroxytryptamine and carbachol respectively. Carbachol also induced a large increase in the level of inositol 1,4,5-trisphosphate in the parotid. Stimulation of brain slices with carbachol induced modest increase in the bis- and tris-phosphate. In all the tissues studied, there was a significant agonist-dependent increase in the level of inositol 1-phosphate. The latter may be derived from inositol 1,4-bisphosphate, because homogenates of the insect salivary gland contain a bisphosphatase in addition to a trisphosphatase. These results suggest that the earliest event in the stimulus–response pathway is the hydrolysis of polyphosphoinositides by a phosphodiesterase to yield inositol 1,4,5-trisphosphate and inositol 1,4-bisphosphate, which are subsequently hydrolysed to inositol 1-phosphate and inositol. The absence of inositol 1:2-cyclic phosphate could indicate that, at very short times after stimulation, phosphatidylinositol is not catabolized by its specific phosphodiesterase, or that any cyclic derivative liberated is rapidly hydrolysed by inositol 1:2-cyclic phosphate 2-phosphohydrolase.

Agonists that interact with receptors to produce a calcium signal are also able to induce the hydrolysis of phosphoinositides. The relationship between calcium signalling and phospholipid metabolism remains very much a controversial issue, mainly because we have relatively little information on the precise mechanism whereby agonists hydrolyse these inositol-containing phospholipids. It has been documented that a number of agonists acting on many different cell types can induce the disappearance of PtdIns (Michell, 1975, 1979; Berridge, 1980, 1981; Michell & Kirk, 1981; Michell *et al.*, 1981; Putney, 1981; Irvine *et al.*, 1982). However, there is also substantial evidence to show that many of these same agonists can also stimulate the disappearance of the polyphosphoinositides represented by PtdIns4*P* and PtdIns(4,5)P_2 (Durell *et al.*, 1968; Abdel-Latif *et al.*, 1977; Griffin &

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins1*P*, *myo*-inositol 1-phosphate; Ins(1,4)P_2, *myo*-inositol 1,4-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-triphosphate.

Hawthorne, 1978; Akhtar & Abdel-Latif, 1980; Kirk *et al.*, 1981; Downes & Michell, 1982; Weiss *et al.*, 1982). The hydrolysis of these polyphosphoinositides can occur through the action of either phosphomonoesterases that liberate P_i or phosphodiesterases that liberate Ins(1,4)P_2 from PtdIns4P and Ins(1,4,5)P_3 from PtdIns(4,5)P_2. In contrast, the phosphodiesterase that hydrolyses PtdIns yields a mixture of inositol 1:2-cyclic phosphate and Ins1P. A study of such inositol phosphates might indicate which of the phosphodiesterases is operating and could thus provide a way of distinguishing whether PtdIns or the polyphosphoinositides are the primary substrates metabolized during the action of agonists such as 5-hydroxytryptamine, acetylcholine, noradrenaline and Substance P. The water-soluble inositol-containing phosphate esters present in various tissues (insect salivary gland, mammalian salivary gland and brain) have been separated and identified by a combination of different chromatographic and ionophoretic techniques that have also been used to provide quantitative measurements of how these metabolites vary during the action of various agonists.

Materials and methods

Incubation of insect salivary glands

Salivary glands were isolated from adult female blowflies and labelled in a medium containing 20μM-*myo*-[2-^{3}H]inositol (sp. radioactivity 9.3Ci/mmol) for 2h. The labelling medium was removed and the glands were rinsed five times with fresh medium over a 30min period. Groups of five glands were incubated at 30°C in 200μl of control medium contained in small glass vials. The medium surrounding the glands was removed and replaced with a further 200μl of medium with or without 10μM-5-hydroxytryptamine. This incubation was terminated by the addition of 1ml of chloroform/methanol (1:2, v/v) or by the addition of 200μl of 15% trichloroacetic acid.

Incubation of parotid gland fragments and brain slices

The dissection and preparation of rat parotid gland fragments and cerebral cortical slices have been described previously (Hanley *et al.* 1980; Berridge *et al.*, 1982). Experiments on both tissues were essentially identical, except that the medium used for parotid gland incubations was a modified Krebs–Ringer bicarbonate containing 10mM-inosine, 0.4mM-adenosine and 5mM-3-hydroxybutyrate. The medium used for the brain slice experiments was Krebs–Ringer bicarbonate containing 10mM-glucose. These solutions were gassed with O_2/CO_2 (19:1) before use and periodically during incubations with tissues.

Samples of each tissue (200μl of gravity-packed slices/ml of incubation medium) were incubated in medium containing 0.32μM-*myo*-[^{3}H]inositol (12.5Ci/mmol) at 37°C for 90min using a gently shaking water bath. The medium containing [^{3}H]inositol was then removed and replaced with fresh solution containing 10mM-unlabelled inositol. The tissues were washed with this solution a further three times, resuspended at the original concentration and the incubation continued for a further 60min at 37°C. This long incubation was essential to 'chase' labelled inositol from the tissues and prevent ligand-stimulated inositol phospholipid labelling (see the Results section). Finally, the slices were washed once more with the unlabelled inositol solution to remove any [^{3}H]inositol that diffused out of the cells during the second incubation period.

The prelabelled tissues were allowed to settle under gravity and 50μl portions were pipetted into Beckman Biovials containing 200μl of the incubation medium. These samples were gassed and capped and then incubated at 37°C for 10min before addition of drugs (10μl) from stock solutions in Krebs–Ringer bicarbonate. The incubations were terminated by the addition of 200μl of 15% trichloroacetic acid.

Assays of inositol 1,4-bisphosphatase and inositol 1,4,5-trisphosphatase

An enzyme preparation was obtained by homogenizing 45 blowfly salivary glands in 600μl of ice-cold buffer containing 60mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and 2mM-Mg^{2+}, pH7.0. Portions (200μl) of this homogenate were incubated with inositol 1,[^{32}P]4-bisphosphate or inositol 1,4,5-[4,5-^{32}P]trisphosphate at 30°C for 5 or 15min. Assays were stopped by addition of an equal volume of 15% (w/v) trichloroacetic acid and the precipitated proteins were removed by centrifugation. In the control, trichloroacetic acid was added to a portion of the gland homogenate before the addition of substrate. Trichloroacetic acid was removed from the supernatant by five extractions with diethyl ether, and the extract was neutralized with 6.25mM-sodium tetraborate before being applied to Dowex-1 columns.

Extraction of water-soluble metabolites

Two different methods were used to extract the water-soluble metabolites. In those cases where the incubations were stopped by the addition of chloroform/methanol, the fluid was transferred to a test tube and the remaining tissue was then homogenized in 200μl of 0.5M-HCl. In two separate experiments we replaced the 0.5M-HCl with distilled water so that the acid-labile inositol 1:2-cyclic

phosphate could be quantitatively extracted. The homogenate was added to the chloroform/methanol extract, as was a further 200μl of water, which was used to wash out the homogenizer. After addition of 200μl of chloroform the tubes were centrifuged. After collecting the upper phase the interface was washed twice with 200μl of an upper phase prepared from chloroform/methanol/0.1M-sodium cyclohexane-1,2-diaminetetra-acetic acid (16:8:5, by vol.). This combined upper phase and washing was then dried and stored at −15°C. This extract was dissolved in 1ml of 6.25mM-sodium tetraborate before being applied to the anion-exchange columns. In those experiments where the incubation was stopped by the addition of ice-cold 15% trichloroacetic acid, the samples were left on ice for 15min. The tubes were centrifuged and the extract was removed and washed five times with diethyl ether. The extract was then neutralized before being applied to the anion-exchange columns.

Separation of water-soluble metabolites by anion-exchange chromatography

The water-soluble extracts were applied to columns containing 1ml of Dowex-1 (X8; formate form; Sigma Chemical Co., London S.W.6, U.K.). The phosphate esters were eluted by the stepwise addition of solutions containing increasing levels of formate as described by Richards *et al.* (1979) and by Downes & Michell (1981). Details of the solutions used are provided in the legend to Fig. 1. A 0.5ml portion of each fraction was taken for liquid-scintillation counting or for total phosphorus determination by the method of Bartlett (1959).

Separation of water-soluble metabolites by high-voltage ionophoresis and paper chromatography

The method used was similar to that described by Dawson & Clarke (1972). For all experiments, the entire paper was cut into 1cm strips and counted for radioactivity by scintillation counting, to ensure both a complete recovery of radioactivity and the detection of all radioactive compounds. Ins(1,4)P_2 was also identified by paper chromatography in n-propanol/conc. NH_3/water (5:4:1, by vol.) as described by Grado & Ballou (1961). Generally, the chromatography was for 48h to ensure a large movement (>20cm) of Ins(1,4)P_2.

Preparation of standards

Glycerophosphoinositol and inositol 1:2-cyclic phosphate were prepared as described by Dawson & Clarke (1972). Ins1P was prepared by the hydrolysis of PtdIns by a brain supernatant. Inositol bisphosphate and inositol trisphosphate of unknown positional isomerism were prepared by acid hydrolysis of PtdIns4P and PtdIns(4,5)P_2 respectively, followed by paper-ionophoretic separation. Also, ^{32}P-labelled Ins(1,4)P_2 and Ins(1,4,5)P_3 were prepared enzymically, either directly from ^{32}P-labelled erythrocyte ghosts as described by Downes *et al.* (1982), or by treating the corresponding radioactive phosphoinositide with a rat brain supernatant.

Results

Identification of water-soluble inositol metabolites in blowfly salivary gland

The water-soluble extract obtained from control or 5-hydroxytryptamine-stimulated blowfly salivary glands contained five distinct ^{3}H-containing peaks when run through an anion-exchange column (Fig. 1). 5-Hydroxytryptamine caused large increases in peaks I, III and IV, with no change in peak II and a small increase in peak V. Five major radioactive spots were also identified when similar extracts were separated by high-voltage ionophoresis. On the basis of previous studies (Dawson & Clarke, 1972) these spots corresponded to inositol, glycerophosphoinositol, Ins1P, Ins(1,4)P_2 and Ins(1,4,5)P_3. This identification of metabolites by ionophoresis was substantiated by using anion-exchange columns to compare the behaviour of standards with the unknown peaks shown in Fig. 1.

For this identification procedure, a large extract was prepared so that similar portions could be compared with the different standards. This procedure provided a way of establishing whether the existence of large amounts of the standard altered the behaviour of the labelled metabolite that exists in trace amounts.

Peak I was established as *myo*-inositol on the basis that it was not retained on the column and that this first peak had a profile identical with that of standard [^{3}H]inositol. Furthermore, ionophoretic experiments revealed that the gland extract contained an uncharged molecule whose mobility was identical with standard inositol.

Peak II was identified as glycerophosphoinositol (Fig. 2*a*). The radioactivity obtained from the gland coincided exactly with standard glycerophosphoinositol. In a separate experiment, a cell extract together with standard glycerophosphoinositol were separated by ionophoresis. The spot corresponding to this substance was then cut out, eluted from the paper and re-run through the anion-exchange column. The result was similar to that shown in Fig. 2(*a*), suggesting that peak II is glycerophosphoinositol. The eluted spot also co-chromatographed exactly with glycerophosphoinositol when run on paper in propanol/NH_3 (Dawson & Clarke, 1972). After digestion with 6M-HCl at 100°C for 18h, all the radioactivity was found to be in a spot corresponding to inositol after chromatography in n-propanol/ethyl acetate/water (24:13:7, by vol.).

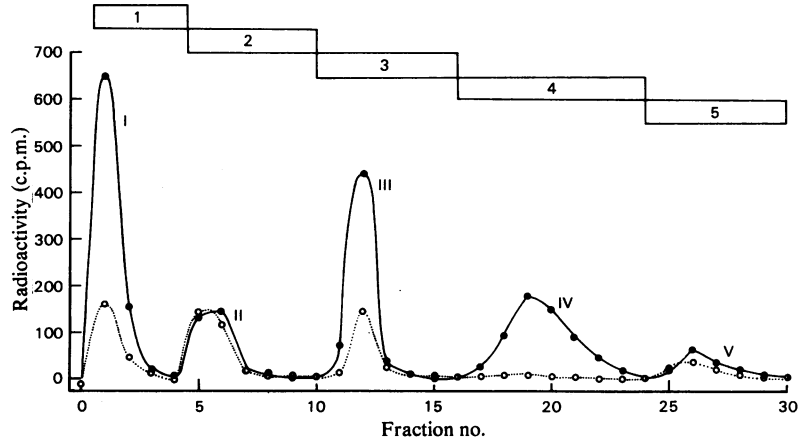

Fig. 1. *Elution profiles of water-soluble extracts of the blowfly salivary gland by anion-exchange columns*
Groups of five salivary glands were incubated with 20 μM-*myo*-[2-^{3}H]inositol for 2 h. The glands were washed and then stimulated for 90 s with 200 μl of 10 μM-5-hydroxytryptamine (●). Controls had 200 μl of vehicle added only (○). The incubations were stopped by adding 1 ml of chloroform/methanol (1:2, v/v). The water-soluble components were applied to Dowex-1 anion-exchange columns and eluted with: (1) distilled water; (2) 5 mM-disodium tetraborate/60 mM-sodium formate; (3) 0.1 M-formic acid/0.2 M-ammonium formate; (4) 0.1 M-formic acid/0.4 M-ammonium formate; (5) 0.1 M-formic acid/1.0 M-ammonium formate.

Peak III has already been identified as Ins*lP* in a previous study (Berridge *et al.*, 1982). Further confirmation is provided in Fig. 2(*b*), where standard Ins*lP* was eluted at the same position as the radioactive metabolite obtained from the salivary glands. A similar correspondence was observed on both ionophoresis and chromatography in propanol/NH_3.

Peak IV was eluted at the same position as standard inositol 1,[^{32}P]4-bisphosphate (Fig. 2c). This peak was also compared with standard inositol bisphosphate prepared by acid hydrolysis of PtdIns4*P*. A cell extract to which standard inositol bisphosphate had been added was firstly separated by paper ionophoresis. One of the unknown radioactive metabolites ran in exactly the same position as the standard inositol bisphosphate. When this spot was eluted and re-run through the anion-exchange columns the radioactivity was eluted in exactly the same position as the standard, again indicating the existence of inositol bisphosphate.

When the radioactivity obtained from peak IV was hydrolysed for 18 h at 100°C in 6 M-HCl, all the radioactivity was recovered in inositol after separation by paper chromatography in n-propanol/ethyl acetate/water (24:13:7, by vol.). The compound in peak IV did not exactly co-chromatograph with the inositol bisphosphate prepared by acid hydrolysis of PtdIns4*P*, when they were run on paper chromatograms in n-propanol/saturated NH_3/water (5:4:1, by vol.) as described by Grado & Ballou (1961). However, such acid hydrolysis randomizes the phosphate groups on the inositol ring, and Grado and Ballou's solvent will separate the different isomers of inositol bisphosphate from one another. That this is so was confirmed in two ways. If peak IV was submitted to 20 min hydrolysis in 1 M-HCl at 100°C, it then chromatographed faster and corresponded to the standard inositol bisphosphate prepared by acid hydrolysis. Secondly, enzymically prepared ^{32}P-labelled Ins(1,4)P_2, when chromatographed in Grado and Ballou's solvent, was found by autoradiography to co-chromatograph exactly with the [^{3}H]inositol-labelled compound from salivary gland, identifying the latter as Ins(1,4)P_2.

Peak V corresponds to standard Ins(1,4,5)P_3. This peak was found to coincide exactly both on anion-exchange columns (Fig. 2*d*) and by ionophoresis with a ^{32}P-labelled Ins(1,4,5)P_3 standard prepared by hydrolysing ^{32}P-labelled PtdIns(4,5)P_2 in erythrocyte membranes (Downes *et al.*, 1982).

In summary, a combination of high-voltage ionophoresis, paper chromatography and anion-exchange chromatography has established that the peaks in Fig. 1 are inositol (peak I), glycerophosphoinositol (peak II), Ins1*P* (peak III), Ins(1,4)P_2 (peak IV) and Ins(1,4,5)P_3 (peak V).

Identification of water-soluble inositol metabolites in extracts from rat parotid gland and brain slices

When extracts obtained from control or

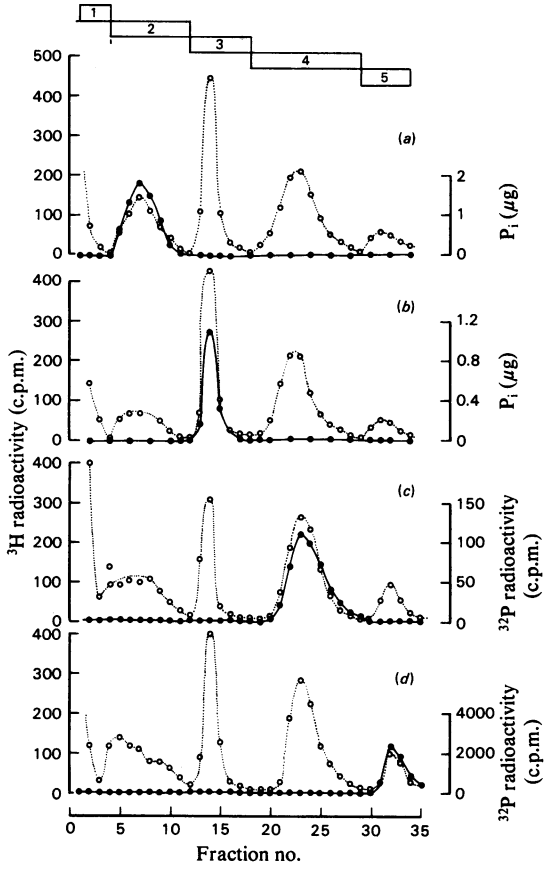

Fig. 2. *Comparison of elution profiles of water-soluble extracts of the blowfly salivary gland with standard glycerophosphoinositol (a), Ins1P (b), Ins(1,[^{32}P]4)P_2 (c) and Ins(1,4,5)[4,5-^{32}P]P_3 (d)*

Groups of five salivary glands were labelled, stimulated with 10μM-5-hydroxytryptamine for 90s and extracted as described in the legend to Fig. 1. Standards (●) were added to the tissue extracts and run through anion-exchange columns using the solutions described in the legend to Fig. 1. Half of each 2ml fraction that was eluted from the columns was counted for ^{3}H radioactivity to identify the position of the different peaks and the other half was used to detect the standards. The standard glycerophosphoinositol (*a*) and Ins1*P* (*b*) were identified by assaying for phosphorus, and the Ins(1,[^{32}P])4P_2 (*c*) and Ins(1,4,5)[4,5-^{32}P]P_3 were detected by Čerenkov counting.

carbachol-treated parotid gland and brain slices were run through a Dowex-1 (formate) column we found five radioactive peaks that corresponded to those obtained using extracts from the blowfly salivary gland. This result was confirmed when components in the extracts were separated by high-voltage ionophoresis. When neutral chloroform/methanol extracts of these tissues were prepared and then subjected to high-voltage ionophoresis we found no evidence for the presence of inositol 1:2-cyclic phosphate.

Table 1. *Recovery of water-soluble inositol metabolites from cell extracts*

A group of 30 glands was labelled with [^{3}H]inositol as described in the Materials and methods section. A water-soluble extract of these glands was used to test the recovery of metabolites from cell extracts. For the experimental set, groups of six unlabelled glands in 200μl of medium were treated with 1ml of chloroform/methanol. A portion of the ^{3}H-labelled cell extract was added to these glands, which were then taken through the usual acid-extraction procedure. For the controls, a portion of the ^{3}H-labelled cell extract was taken through the same procedure but without any unlabelled glands. The results represent the average of two separate samples that closely agreed with each other.

	^{3}H radioactivity (c.p.m.)				
	Inositol	Glycero-phospho-inositol	Ins1*P*	Ins(1,4)P_2	Ins(1,4,5)P_3
Control	1428	976	750	1141	289
Experimental	1481	967	660	1098	295

Efficiency of the procedure for extracting metabolites from tissues

As a prelude to a quantitative analysis of how these water-soluble metabolites vary during the action of agonists, tests were carried out on the efficiency of the extraction procedure. A bulk extraction containing ^{3}H-labelled inositol metabolites was prepared from the blowfly salivary gland and portions were added to unlabelled glands and taken through the normal extraction procedure. Similar portions taken through the same procedure but in the absence of unlabelled glands served as controls. All the metabolites were recovered completely apart from a small reduction in the level of Ins1*P* (Table 1).

Changes in the level of inositol and inositol phosphates during stimulation of the blowfly salivary gland with 5-hydroxytryptamine

As shown in Fig. 1, 5-hydroxytryptamine induced large increases in the levels of radioactivity appearing in inositol (peak I), Ins1*P* (peak III) and Ins(1,4)P_2 (peak IV). A quantitative analysis of these changes is presented in Table 2. The samples obtained from control and stimulated glands were analysed by both anion-exchange chromatography and paper ionophoresis. Both techniques revealed

Table 2. *Effect of 5-hydroxytryptamine on the level of water-soluble inositol metabolites of the blowfly salivary gland*
Groups of 10 salivary glands, which had been labelled with [^{3}H]inositol, were incubated for 1 min either in the absence or in the presence of 10 μM-5-hydroxytryptamine. The incubation was stopped by the addition of chloroform/methanol. Water-soluble metabolites were isolated and analysed either by anion-exchange chromatography or by paper ionophoresis. Results are expressed as means ± S.E.M. for the numbers of experiments given in parentheses. Statistical significance was calculated by using Student's *t* test. *$P < 0.01$. Only a part of each tube of anion-exchange column effluent was counted for radioactivity to avoid overloading the scintillation cocktail. Abbreviation used: 5-HT, 5-hydroxytryptamine.

		^{3}H radioactivity (c.p.m./gland)				
Technique	Treatment	Ins	Glycerophospho-inositol	Ins1*P*	Ins(1,4)P_2	Ins(1,4,5)P_3
Anion-exchange	Control	158 ± 13 (6)	599 ± 123 (6)	109 ± 18 (5)	28 ± 3 (6)	62 ± 16 (6)
	5-HT	396 ± 45 (6)*	612 ± 57 (5)	208 ± 8 (5)*	269 ± 18 (5)*	87 ± 11 (5)
Ionophoresis	Control	238 ± 119 (5)	364 ± 235 (5)	245 ± 123 (5)	52 ± 50 (6)	20 ± 17 (6)
	5-HT	758 ± 160 (6)*	576 ± 148 (6)	981 ± 317 (6)*	646 ± 241 (6)*	26 ± 16 (6)

Table 3. *The effect of Ca^{2+}-mobilizing agonists on the levels of water-soluble inositol metabolites in rat parotid glands*
Parotid gland slices were prelabelled with [^{3}H]inositol and washed as described in the Materials and methods section. Samples of this tissue slice preparation were incubated with the agonists and antagonists shown for 10 min at 37°C. The incubations were stopped by adding trichloroacetic acid and [^{3}H]inositol-labelled metabolites in the soluble fraction were analysed by anion-exchange chromatography on Dowex-1 (formate form) columns. Results are means ± S.E.M. of triplicate incubations and are for one of three experiments that gave essentially similar results. *$P < 0.02$ (Student's *t* test).

	Radioactivity in inositol metabolites (d.p.m.)				
Drug additions	Inositol	Glycerophospho-inositol	Ins1*P*	Ins(1,4)P_2	Ins(1,4,5)P_3
None	104 800 ± 18 400	250 ± 20	630 ± 80	250 ± 24	800 ± 30
Carbachol (1 mM)	157 200 ± 26 800	439 ± 60	9450 ± 960*	11 050 ± 980*	5650 ± 310*
Carbachol (1 mM + atropine (10 μM)	97 780 ± 8330	230 ± 13	590 ± 90	390 ± 80	950 ± 70
Phenylephrine (0.1 mM)	113 100 ± 6000	270 ± 12	1440 ± 90*	1390 ± 70*	2330 ± 120*
Substance P (2 μM)	106 000 ± 10 200	270 ± 16	1190 ± 80*	1360 ± 120*	1830 ± 120*

Table 4. *The effect of carbachol on the levels of water-soluble inositol metabolites in rat brain slices*
Rat brain slices were treated exactly as described in the legend to Table 3. Results are means ± S.E.M. of four replicate samples in a single experiment, one of three that gave essentially similar results. Statistical significance was assessed by using Student's *t* test. * Indicates a significant difference from control value ($P < 0.02$).

	Radioactivity in inositol metabolites (d.p.m.)				
Drug additions	Inositol	Glycerophospho-inositol	Ins1*P*	Ins(1,4)P_2	Ins(1,4,5)P_3
None (control)	231 400 ± 5220	914 ± 15	1600 ± 170	360 ± 24	320 ± 31
Carbachol (1 mM)	257 960 ± 15 830	984 ± 65	2180 ± 150	600 ± 57*	460 ± 11*

that the major change that occurred upon stimulation with 5-hydroxytryptamine was a large increase in the level of Ins(1,4)P_2 (Table 1). There was also a significant increase in the level of Ins1*P* and free inositol. There was no significant difference in the level of either glycerophosphoinositol or Ins(1,4,5)P_3. When glands were stimulated over shorter time periods, there was a large increase in the level of Ins(1,4,5)P_3 (results not shown). In two separate experiments we specifically looked for radioactivity in inositol 1:2-cyclic phosphate (see the Materials and methods section) but could not find any in either control or 5-hydroxytryptamine-stimulated glands.

Changes in the levels of ^{3}H-labelled metabolites during stimulation of parotid gland slices with Ca^{2+}-mobilizing agonists

When parotid gland slices are labelled with [^{3}H]inositol and then stimulated with carbachol there is an increase in the rate of incorporation of the radioisotope into PtdIns (Hanley *et al.*, 1980). This enhanced labelling was prevented if the pre-labelled glands were first extensively washed in a medium containing 10mM unlabelled inositol as described in the Materials and methods section (results not shown). However, if the time of the incubation with the unlabelled inositol was reduced from 60 min to 30 min, then a small PtdIns-labelling response to carbachol still occurred.

Separation of ^{3}H-labelled metabolites on Dowex-1 (formate) columns was the most convenient chromatographic technique for routine quantitative analysis. The results of such separations of the components in extracts from parotid gland slices that had been stimulated with the Ca^{2+}-mobilizing agonists carbachol (acting at muscarinic receptors), phenylephrine and Substance P are shown in Table 3. After 10 min exposure to carbachol (1 mM), there were large increases in the levels of radioactivity found in the peaks corresponding to Ins1*P*, Ins(1,4)P_2 and Ins(1,4,5)P_3. The biggest change was found in the Ins(1,4)P_2 fraction, which increased about 44-fold. There was only a modest increase in the glycerophosphoinositol fraction and the apparent increase in the level of [^{3}H]inositol was not significantly different from the control. All of the changes were blocked by 10μM-atropine.

The effects of phenylephrine and Substance P were quantitatively much smaller, but qualitatively similar to those of carbachol. Once again the most prominent increase, with both those agonists, was in the Ins(1,4)P_2 fraction, with significant rises in the levels of Ins1*P* and the trisphosphate. We could not detect a change in the levels of glycerophosphoinositol or free inositol.

Homogenates of parotid gland, like the blowfly salivary gland (see below), rapidly hydrolysed ^{32}P-labelled Ins(1,4,5)P_3 (results not shown). Therefore these results are most easily reconciled with the notion that each of the Ca^{2+}-mobilizing agonists stimulates a polyphosphoinositide phosphodiesterase that generates Ins(1,4,5)P_3 and/or Ins(1,4)P_2. Ins1*P* could then arise by stepwise hydrolysis of the polyphosphates as described for the blowfly. The results do not entirely preclude the possibility that some Ins1*P* might be formed directly from PtdIns.

Changes in the levels of ^{3}H-labelled metabolites during stimulation of rat brain slices with carbachol

Table 4 shows that carbachol induced modest increases in the levels of Ins(1,4)P_2 and the inositol trisphosphate. In the experiment shown we did not detect a significant increase in Ins1*P* but this compound tended to accumulate with time and it usually rose significantly when the incubation time

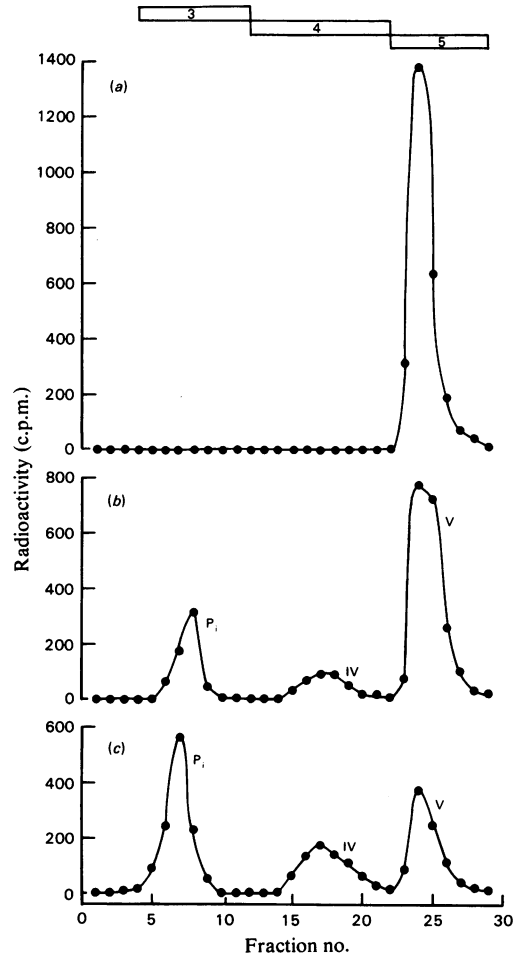

Fig. 3. *Inositol trisphosphate phosphomonoesterase activity in a homogenate of the blowfly salivary gland*
An enzyme preparation was obtained by homogenizing 45 salivary glands in 600μl of medium containing Hepes (50mM) and Mg^{2+} (2mM) at pH7.0. Of this enzyme preparation 200μl was incubated with Ins(1,4,5)[4,5-^{32}P]P_3 for different times at 30°C. The incubation was stopped by the addition of 200μl of ice-cold 15% trichloroacetic acid. After removing trichloroacetic acid, the samples were loaded on the columns and eluted by using solution (3) for removing [^{32}P]P_i, solution (4) for removing Ins(1,[^{32}P]4)P_2 (peak IV) and solution (5) for removing Ins(1,4,5)[4,5-^{32}P]P_3 (peak V) as described in the legend to Fig. 1. (*a*) Control (200μl of trichloroacetic acid was added to 200μl of enzyme extract before addition of substrate); (*b*) 5min incubation; (*c*) 15min incubation.

was increased to 30min (results not shown). Although the results for brain slices are not so startling as those for the mammalian salivary glands, they do suggest that a similar, if not identical, enzyme mechanism underlies the action of muscarinic agonists in the rat and of 5-hydroxytryptamine in the blowfly.

Inositol 1,4-bisphosphatase and inositol 1,4,5-trisphosphatase activity in insect salivary gland

A homogenate of the insect salivary gland was able to degrade inositol 1,4,5-[4,5-^{32}P]trisphosphate to inositol 1,[^{32}P]4-bisphosphate (Fig 3). The other product formed was [^{32}P]P_i, which appeared in the first peak. At 15min, the [^{32}P]P_i peak was considerably larger than the Ins(1,[^{32}P]4)P_2 peak, suggesting that the latter had also been degraded by an inositol 1,4-bisphosphatase. However, such a difference could arise if radioactivity was distributed unevenly between the 4- and 5-positions on the inositol ring. A more direct demonstration of the existence of an inositol 1,4-bisphosphatase was obtained by showing that [^{32}P]P_i was liberated when these gland extracts were incubated with Ins(1,[^{32}P]4)P_2 (M. J. Berridge, R. M. C. Dawson, C. P. Downes, J. P. Heslop & R. F. Irvine, unpublished work). As there was no evidence for any inositol 4-[^{32}P]phosphate, even when assays were performed in the presence of 10mM-Li^+ to inhibit inositol 1-phosphatase activity (Hallcher & Sherman, 1980), we conclude that the inositol 1,4-bisphosphatase removes phosphate specifically from the 4-position on the inositol ring. Ins1P would not be detected in our elution profiles since the substrate was prepared from red blood cells and thus carries [^{32}P]phosphate at the 4- and 5-positions but not at the 1-position (Downes & Michell, 1981).

Discussion

Cell membranes contain three phosphoinositides that may play some undefined role in the responsiveness of cells to certain agonists. In mass terms the major component is PtdIns some of which can be phosphorylated by specific kinases to produce the two polyphosphoinositides [PtdIns4P and PtdIns(4,5)P_2]. These polyphosphoinositides can be converted back into PtdIns by means of phosphomonoesterases. These lipids are thus readily interconvertible by the addition or removal of phosphate at the 4- and 5-positions of the inositol ring. Interest in these phosphoinositides with regard to cellular control mechanisms stems from the fact that they are rapidly hydrolysed when cells respond to a variety of hormones or neurotransmitters (Michell, 1975, 1979; Berridge, 1980, 1981; Michell & Kirk, 1981; Michell *et al.*, 1981; Putney, 1981; Irvine *et al.*, 1982). When such agonists interact with the appropriate receptor, there is a rapid disappearance of these phosphoinositides. Although most attention has been focused on PtdIns, there is now considerable evidence to show that there is also a rapid removal of the polyphosphoinositides (Downes & Michell, 1982). Indeed, the latter are broken down considerably faster than PtdIns when liver cells are stimulated with vasopressin (Kirk *et al.*, 1981). Although it has been clearly established that agonists can stimulate the breakdown of all the phosphoinositides, the precise biochemical pathways responsible for this reduction in lipid level are not clear. Since all these phosphoinositides are readily interconvertible, there are a number of ways whereby an agonist could induce such a breakdown of all three forms. For example, a reduction in the level of PtdIns could occur through an increase in its phosphorylation to PtdIns4P or, as seems more likely, an increase in its hydrolysis by a phosphodiesterase. Likewise, the polyphosphoinositides could decline either by being dephosphorylated back to PtdIns by means of phosphomonoesterases or by being hydrolysed by a phosphodiesterase. It is not possible, therefore, to determine which pathway is being used simply by studying the rate at which these phospholipids are being degraded.

Another approach is to study the water-soluble products that are released when these phosphoinositides are hydrolysed by the relevent phosphodiesterases. The water-soluble products are sufficiently unique as to provide a chemical signature that should identify which phosphoinositide is the primary substrate for the receptor mechanism. For example, the hydrolysis of PtdIns by its phosphodiesterase yields a mixture of inositol 1:2-cyclic phosphate and Ins1P; PtdIns4P gives Ins(1,4)P_2; and PtdIns(4,5)P_2 will produce Ins(1,4,5)P_3. The existence of such inositol phosphates was first described by Durell *et al.* (1968), who showed that a crude mitochondrial fraction of brain contained Ins1P, Ins(1,4)P_2 and traces of the trisphosphate. Acetylcholine stimulated a small increase in the levels of Ins1P and Ins(1,4)P_2. An increase in these two inositol phosphates has also been described in guinea-pig synaptosomes after treatment with ionophore A23187 (Griffin & Hawthorne, 1978). A slightly different pattern was described in rabbit iris smooth muscle, where acetylcholine stimulated an increase in the release of Ins1P and Ins(1,4,5)P_3 with no change in the level of the bisphosphate (Akhtar & Abdel-Latif, 1980). In the present study on brain and on insect and mammalian salivary gland, we have identified four major inositol phosphates [glycerophosphoinositol, Ins1P, Ins(1,4)P_2 and Ins(1,4,5)P_3]. In a previous study on the insect salivary gland, Fain & Berridge (1979), by using a paper-chromatographic separation technique, reported the existence of

glycerophosphoinositol, Ins1*P* and inositol 1:2-cyclic phosphate. The presence of the cyclic phosphate is of particular significance because its existence would suggest that PtdIns was being degraded by its specific phosphodiesterase as part of the receptor mechanism. However, the more sophisticated separation techniques described in the present paper have failed to detect any inositol 1:2-cyclic phosphate, but instead have detected the existence of Ins(1,4)P_2 and Ins(1,4,5)P_3. It seems likely that the radioactive material previously identified by Fain & Berridge (1979) as inositol 1:2-cyclic phosphate may have been contaminated with Ins(1,4)P_2. The identification of these various products has been verified by using a variety of separation techniques and a routine procedure has been developed for measuring these inositol phosphates in tissue extracts.

A characteristic feature of the responsiveness of the insect salivary gland and mammalian parotid gland to agonists was the very large increase in the inositol phosphates derived from the polyphosphoinositides. In the insect salivary gland, there was a 10-fold increase in the level of Ins(1,4)P_2 after stimulation with 5-hydroxytryptamine for 1 min. Agonists such as carbachol induced very large increases in the levels of Ins(1,4)P_2 and Ins(1,4,5)P_3 in the parotid. Such large increases in the levels of these two inositol phosphates would seem to suggest that one of the primary biochemical actions of the agonist is to stimulate the hydrolysis of the polyphosphoinositides by a phosphodiesterase. A previous study on the parotid has already described a large decrease in the level of ^{32}P-labelled PtdIns(4,5)P_2 upon stimulation with methacholine, Substance P or adrenaline (Weiss *et al.*, 1982). An increase in the appearance of Ins(1,4,5)P_3 is thus consistent with the idea that the agonist is acting to stimulate the hydrolysis of a polyphosphoinositide through a phosphodiesterase rather than through a phosphomonoesterase pathway. However, the picture is complicated by the fact that these agonists also increased the level of Ins1*P* and free [^{3}H]-inositol, which is presumably produced from Ins1*P* by phosphomonoesterase action. This Ins1*P* could be formed directly by the breakdown of PtdIns or it might arise from the action of phosphomonoesterase on the Ins(1,4)P_2 and In(1,4,5)P_3. Although the failure to detect inositol 1:2-cyclic phosphate could suggest that the latter pathway operates, it is known that many tissues contain an active inositol 1:2-cyclic phosphate 2-phosphohydrolase (Dawson & Clarke, 1972), which could rapidly convert the cyclic derivative into Ins1*P*.

Not much is known about the phosphomonoesterases that carry out the stepwise dephosphorylation of Ins(1,4,5)P_3 to inositol. The first enzyme in the sequence, an Ins(1,4,5)P_3 phosphomonoesterase, has been characterized in red cell membranes (Downes *et al.*, 1982). A characteristic feature of this enzyme is that it specifically removes the phosphate at the 5-position to yield Ins(1,4)P_2. A crude homogenate of the insect salivary gland was capable of removing phosphate from the 4- and 5-positions to yield Ins1*P*. Since there was no accumulation of Ins4*P*, it seems likely that the bisphosphatase is specific for the phosphate carried on the 4-position of the inositol ring. The final step in this pathway is the conversion of Ins1*P* into free inositol by the inositol 1-phosphatase. Inhibition of this enzyme by Li^+ leads to very large accumulations of Ins1*P* in a number of tissues (Berridge *et al.*, 1982). These phosphomonoesterases are thus quite specific in that the trisphosphatase removes the 5-phosphate, the bisphosphatase removes the 4-phosphate, and the last enzyme in the sequence removes phosphate from the 1-position.

An interesting feature of these enzyme studies was that the inositol trisphosphatase that removed the phosphate at the 5-position appeared to be somewhat more active than the bisphosphatase that removes the phosphate from the 4-position. If this difference in enzyme activity also occurs *in vivo*, it would explain why stimulation of the insect gland with 5-hydroxytryptamine induced a large accumulation of Ins(1,4)P_2 with little change in the level of Ins(1,4,5)P_3, which is presumably rapidly degraded. When the insect salivary gland was stimulated with 5-hydroxytryptamine over time periods of less than 1 min, there was a transient increase in the level of Ins(1,4,5)P_3 (M. J. Berridge, unpublished work).

The large accumulations of Ins(1,4)P_2 and Ins(1,4,5)P_3 thus indicate that agonists may act by initiating the hydrolysis of the polyphosphoinositides through a specific phosphodiesterase. These inositol phosphates are then dephosphorylated to inositol through a series of phosphomonoesterases. Such a mechanism implies that the agonist-dependent decline in the level of PtdIns that has previously been described in many tissues could arise indirectly, as this lipid is phosphorylated to replace the polyphosphoinositides being consumed by the receptor mechanism. In other words, PtdIns would be removed through the action of a kinase rather than through a phosphodiesterase. On the basis of this model, the increase in Ins1*P* is the result of a bisphosphatase acting on the Ins(1,4)P_2 arising from the polyphosphoinositides. However, the data in the present paper cannot preclude the possibility that some Ins1*P* may be derived from direct action on PtdIns of its specific phosphodiesterase, which exists in high concentrations in all tissues so far examined. A kinetic analysis of the early changes that occur in these inositol phosphates upon stimulation will be required to throw more light on

which of these phosphoinositides is the primary substrate for the receptor mechanism.

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